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Double-stranded siRNA targeted to the huntingtin gene does not induce DNA methylation

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Abstract

RNA interference is an evolutionarily conserved mechanism of post-transcriptional gene silencing. Small interfering RNAs (siRNA) of 21–23 nucleotides generated from processing double-stranded RNA (dsRNA) by ribonuclease III, Dicer, are widely used for selective sequence-specific gene silencing in a broad range of organisms. In plants, siRNA is associated with de novo RNA-directed DNA methylation (RdDM) at the homologous target genomic region. To examine RdDM in somatic cells, human glioblastoma cell lines were treated with siRNAs homologous to the human huntingtin gene responsible for Huntington's disease. Methylation of CpG dinucleotides in the plasmid vectors expressing the dsRNAs and homologous genomic region was investigated by bisulfite-mediated genomic sequencing. Target regions of the siRNA in the huntingtin gene showed no significant change in the pattern of DNA methylation, and no CpG methylation was observed on the plasmid vectors. These results indicate that siRNA is not directly linked to DNA methylation at the target huntingtin genomic locus in human cells.

Keywords: Bisulfite sequencing; CpG dinucleotides; De novo DNA methylation; RNA interference; Huntington's disease; Neuronal cell culture; Epigenetic modification; Post-transcriptional gene silencing; Plant genetics

RNA interference (RNAi) was first identified in the nematode *Caenorhabditis elegans* [1,2] and is currently being used for gene-specific, post-transcriptional silencing in other organisms including plants, fungi, insects, and mammals [3–5]. In RNAi, double-stranded RNA (dsRNA) is processed to small interfering RNA (siRNA) 21–23 nt in length by the cellular ribonuclease III, Dicer [6,7]. This siRNA is known to target and selectively degrade homologous mRNAs incorporated into RNA-induced silencing complexes (RISC) [8]. In plants, RNAi is referred to as post-transcriptional gene silencing (PTGS), and involves the processing of small dsRNAs into siRNAs by an evolutionarily conserved manner (reviewed in [9]). Interestingly, expression of dsRNA in plants can also induce epigenetic changes, including de novo DNA methylation of genomic sequence that is homologous to the dsRNA [10–12]. It has been reported that the siRNAs which correlate with DNA methylation at genomic target regions in plants are 24–26 nt in length [12]. Conversely, the shorter (21–22 nt) siRNAs correlate with mRNA degradation, but not with the observed methylation of homologous DNA. Thus, siRNA in plants could play a dual role both in PTGS and in transcriptional gene silencing (TGS) via methylation. The close connection between DNA methylation and siRNA in plants is further supported by the observation that genes, such as RDR2, DCL3, and AGO4, which participate in PTGS, are also involved in the de novo methylation of DNA in plant chromosomal loci [13].

The conserved pathway of RNAi or PTGS mediated by dsRNA in animals and plants suggests that the RNA-directed DNA methylation (RdDM) observed in

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plants might also exist in animals. In the present study, we investigated the methylation status of CpGs at the genomic regions in the human huntingtin gene to determine if dsRNAs modify the endogenous DNA methylation pattern in mammalian cells. Our results indicated that neither the target DNA sequences of the dsRNA nor the flanking genomic sequences revealed changes in CpG methylation status. In addition, no DNA methylation was observed in the plasmid vector expressing the dsRNA. These results suggest that RdDM may be a plant-specific phenomenon and that siRNAs targeted to mammalian genes do not induce significant changes in DNA methylation at the target site.

Materials and methods

Double-stranded RNA constructs. Two dsRNA constructs pH3 and pH5 were made against the human huntingtin gene by cloning inverted repeat (IR) sequences into the psiRNA-hH1-zeo vector (InvivoGen, San Diego, CA, USA). The dsRNA template consisted of a 21 bp target sequence derived from the huntingtin gene, and a 7 bp linker (5′-TCAAGAG-3′) attached to the reverse/complementary target sequence for transcription of the short-hairpin (shRNA) dsRNA. Specifically, the H3 IR sequence 5′-ACCGCCATGGCGACCCTGGAATCAA GAGTTCCAGGGTCGCCATGGCGGT-3′ corresponded to nts 310–330 of the huntingtin mRNA sequence (GenBank Accession No. L12392), as underlined. Similarly, the H5 IR 5′-ATCTTCCAAGG TTACAGCTCGTCAAGAGCGAGCTGTAACCTTGGAAGAT-3′ sequence was derived from nts 806 to 826 of the huntingtin mRNA, as indicated by underline. The target sequence of H3 spans the ATG translation initiation codon, while the H5 construct is completely within the fourth exon of the human huntingtin gene. Each IR sequence and complementary oligonucleotide was synthesized with *Bbs*I restriction endonuclease linkers (Integrated DNA Technologies, Coralville, IA, USA) and cloned into the *Bbs*I digested psiRNA-hH1-zeo vector. The complete IR sequence of the pH3 and pH5 constructs was verified by DNA sequencing.

Cell culture and transfection with dsRNA constructs. Two human glioblastoma cell lines, U-87 (ATCC # HTB-14) and U-118 (ATCC # HTB-15), were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA), penicillin, streptomycin sulfate, and amphotericin B at 1 U, 1 μg and 0.25 μg/ml, respectively, under 5% CO_2 at 37 °C. Cells plated at 3×10^5/35 mm dish were transfected with 1 μg of either pH3 or pH5 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended protocol. Each cell line was also transfected in parallel with 1 μg of a control plasmid that expresses the red fluorescent protein, DsRed2.

Bisulfite genomic sequencing. Seven days after transfection, the cells were harvested by scraping and genomic DNA was isolated by phenol–chloroform extraction followed by ethanol precipitation [14]. The genomic DNA was subsequently subjected to bisulfite treatment as described previously [15–17]. Briefly, genomic DNA was digested with a proper set of restriction endonucleases to decrease the fragment size to ensure efficient denaturation. Restriction endonucleases whose recognition sites were located in the DNA region of investigation were avoided. Following digestion and purification, 2 μg of genomic DNA was denatured in 0.3 M NaOH for 20 min at 42 °C. A mixture of sodium bisulfite, urea, and hydroquinone was added to the denatured DNA to final concentrations of 5.8 M urea, 3.7 M sodium bisulfite, and 8.8 mM hydroquinone and incubated for 12 h at 55 °C, covered by aluminum foil to prevent light exposure. The DNA was then purified using QIAEXII resin (Qiagen, Valencia, CA, USA), treated with 0.3 M NaOH for 20 min at 37 °C, and precipitated overnight at −20 °C after addition of ammonium acetate to a final concentration of 3 M, 1 μg tRNA carrier (Sigma–Aldrich, St. Louis, MO, USA), and 3 volumes of ethanol. Following centrifugation, the DNA was washed with 70% ethanol, air-dried, and dissolved in 30 μl H_2O. Approximately 50 ng of bisulfite-treated DNA was used as the template for PCR amplification of the target regions using Expand Hi-Fidelity polymerase (Roche Diagnostics, Indianapolis, IN, USA), as specified by the manufacturer. PCR primers were designed on the basis of predicted DNA sequences. PCR primers used for the amplification of the specific DNA regions after bisulfite treatment were as follows: pMB1 origin of replication (ori) and the region of the psiRNA-hH1-zeo vector encoding the dsRNA template forward, 5′-ATTGGTAGTAGTTATTGGTAA TAGGAT-3′ and reverse, 5′-ACTAACACACATTCCACAACTA ATTC-3′; the region of the psiRNA-hH1-zeo vector containing a CpG island within the human EF1-HTLV promoter which drives expression of the zeo-resistance gene forward, 5′-TATAGTTTTATTAGTTA GTGGGTAGAG-3′ and reverse, 5′-AACTTAACCATAATAAC CCTCCTATAA-3′; H3 huntingtin genomic exon 1 target region forward, 5′-GGTTAGGGTTGTTAATTATGTTGGT-3′ and reverse, 5′-AACCTTCATCAACTTTTCCAAAATC-3′; the endogenous promoter region of the human huntingtin gene forward, 5′-GGGAGG GTAAATTTTAAGGTTATTT-3′ and reverse, 5′-CAAATTCTA CCTCACACAACAAAAC-3′; and H5 huntingtin genomic target region forward, 5′-TTAGAATTAGTGATGGGATGTGTT-3′ and reverse, 5′-CAAACATAAACCACTATACCCTAAC-3′. The region of psiRNA-hH1-zeo vector subjected to bisulfite sequencing includes a part of pMB1 ori, the human H1 promoter, and regions encoding the dsRNAs. Following purification of the PCR amplicons, they were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). At least eight bisulfite PCR clones spanning the target regions derived from two different experiments in each cell line were sequenced. We determined the original methylation status of each CpG dinucleotide within a region by comparing the sequences from control untreated DNA with those of the cloned PCR products from the bisulfite-treated DNA. Only data from bisulfite PCR clones showing complete C-to-T conversion by bisulfite treatment at Cs not located in CpG dinucleotides throughout a region were used.

Results and discussion

DNA methylation of the plasmid vector for the dsRNA templates

To explore the possibility that *cis* DNA methylation is induced by the transcription of siRNA, we investigated if dsRNA expression would cause de novo CpG methylation of the plasmid vectors. Two different plasmids, pH3 and pH5, expressing dsRNAs homologous to different regions in the human huntingtin gene were constructed. Both pH3 and pH5 express dsRNAs targeted to 21 bp huntingtin gene sequences spanning the ATG initiation codon (nts 310–330, GenBank Accession No. L12392), and exon 4 (nts 806–826), respectively. Seven days following transfection with either the pH3 or pH5 construct, or a control plasmid, DNA was isolated from human glioblastoma cell lines U-87 or U-118 and analyzed by bisulfite-mediated sequencing [17] to identify the methylation pattern of the episomal plasmid construct. A region containing part of the CpG-rich pMB1 ori, the human H1 RNA polymerase III promoter [18], and the template encoding the dsRNA was

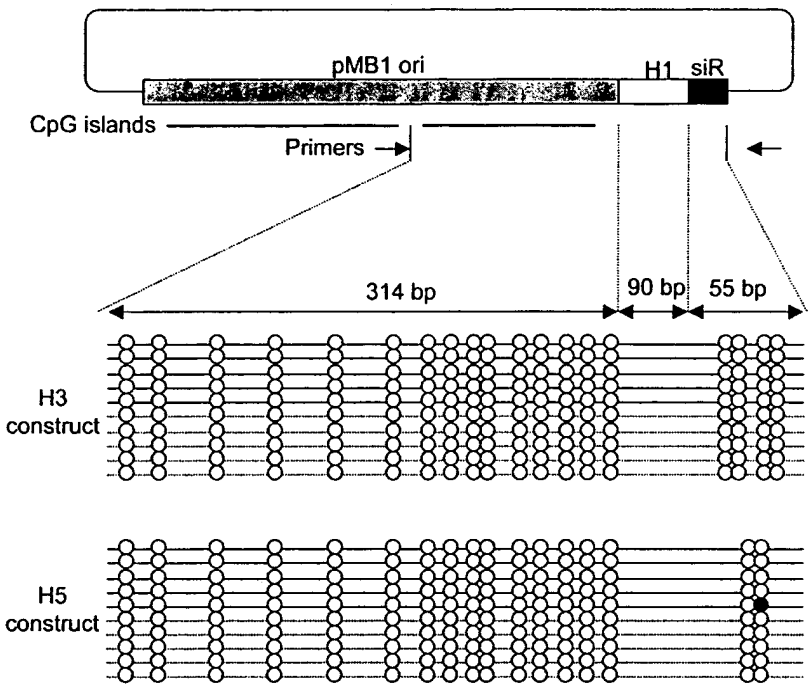

Fig. 1. DNA methylation on the plasmid vector for dsRNA. Schematic circular map of the dsRNA vector is shown at top. The region upstream of the dsRNA template sequence (siR, black box) contains the pMB1 origin of replication (ori) (gray box) and the human H1 promoter (H1, open box). Two CpG islands located within the pMB1 ori are represented by thick lines and the positions of the bisulfite PCR primers are indicated by arrows, below the map. The region investigated by bisulfite sequencing encompassed 314 bp sequence of a CpG island in the pMB1 ori, the 90 bp human H1 promoter (H1), and the 55 bp dsRNA template sequences. Below the diagram, each line represents a bisulfite PCR clone where the methylated and unmethylated CpG dinucleotides are depicted as filled and open circles, respectively. Solid lines and dotted lines represent bisulfite clones derived from the U-87 and U-118 cell lines, respectively. Bisulfite PCR clones derived from cells transfected with pH3 or pH5 are grouped together with the relevant construct indicated at left.

selected for bisulfite-mediated sequence analysis (Fig. 1). The methylation pattern of the selected DNA region in the episomal constructs was established by sequencing PCR clones derived from bisulfite-treated DNA isolated from the transfected cell lines. The observed DNA methylation profiles clearly showed no apparent CpG methylation of this plasmid region by bisulfite sequencing. This complete absence of DNA methylation was observed for both the pH3 and pH5 constructs in each human glioblastoma cell line.

We examined another region within the pH3 and pH5 constructs by bisulfite-mediated sequencing. Specifically, we determined the DNA methylation pattern of a CpG island in the human EF1-HTLV promoter, which drives expression of the Zeocin resistance gene in the constructs. Similar to the CpG island region 5′ to the sequence encoding the dsRNA, there was no discernable DNA methylation in this region of the human EF1-HTVL promoter on either the pH3 or pH5 construct for both the U-87 or U-118 cell lines (data not shown). Taken together, these data suggest that RNA polymerase III-mediated expression of dsRNA from synthetic inverted repeat (IR) structures does not induce de novo DNA methylation of either the dsRNA template, or other regions encompassing CpG islands in the plasmid constructs.

It has been reported that transcription of a specific antisense RNA (LUC7L) caused DNA methylation of a CpG island in a neighboring α2-globin gene in humans as well as in a mouse model, thereby silencing the gene on the sense strand [19]. Interestingly, this phenomenon was dependent on transcription of the antisense RNA in *cis*, and was independent of the promoter used to express the antisense RNA. In our siRNA system, the dsRNA targeted to the huntingtin gene was linearly transcribed in *cis* from an IR sequence to form dsRNA from complementary sequences of the IR. Thus, in animals, DNA methylation seems to be more closely tied to the transcription of antisense RNAs, which is observed in mammalian imprinted genes (reviewed in [20]) than to the existence of dsRNA. In fact, this episomal plasmid system could be easily adapted to determine if transcription of an antisense RNA from an episomal plasmid construct induces de novo DNA methylation silencing via the sense strand.

CpG methylation at the target genomic site of H3 dsRNA construct

To determine if dsRNA induces de novo DNA methylation at the homologous target region of the human genome similar to RdDM in plants, we

examined the CpG methylation status of the genomic sequence directly 5′ to the H3 dsRNA construct target in exon 1 (Fig. 2). The region of the huntingtin gene homologous to the H3 dsRNA is also located approximately 35 bp 5′ of the characteristic CAG repeats of the Huntington's disease gene [21]. A 250 bp CpG island ending immediately 5′ of the H3 target sequence was identified by computer analysis within the first exon using MethPrimer [22]. We determined the methylation pattern of the CpG island as well as the H3 target genomic sequence using bisulfite-mediated genomic sequencing.

Genomic DNA isolated from the human glioblastoma cell lines transfected with either the DsRed2 expressing control construct or with pH3 was subjected to bisulfite-mediated sequencing. Genomic DNA from untransfected control cells was also analyzed to establish the native CpG methylation profile of the target region. The chromosomal DNA region examined by methylation analysis encompassed 32 CpGs, with the most 3′ CpG being present in the H3 genomic DNA target. The methylation profile of these 32 CpGs indicated that the H3 target genomic site and its flanking CpG island are almost completely devoid of methylation irrespective of cell treatment (Fig. 2). Moreover, there was no observed difference in CpG methylation of this DNA region between the U-87 and U-118 human glioblastoma lines. The expression of the pH3 dsRNA was correlated with a >50% reduction in both cellular huntingtin mRNA and protein levels in the transfected cells (data not shown). This result suggests that the DNA methylation directed by dsRNA in plants is not recapitulated in this mammalian cell culture system.

To determine if changes in DNA methylation of non-transcribed flanking regions were induced by the H3 or H5 dsRNA, the endogenous promoter region of the huntingtin gene was also studied by identical methods. The DNA methylation pattern of a ~600 bp CpG island located in the promoter region immediately upstream of transcription start site was analyzed by bisulfite-mediated sequencing. The 61 CpGs contained within the huntingtin promoter CpG island were entirely free of methylation in both cell lines following transfection with either pH3, pH5, or the DsRed2 control plasmid (data not shown). This implies that dsRNA does not induce noticeable DNA methylation either in the huntingtin exon 1 target region or in the nearby huntingtin promoter.

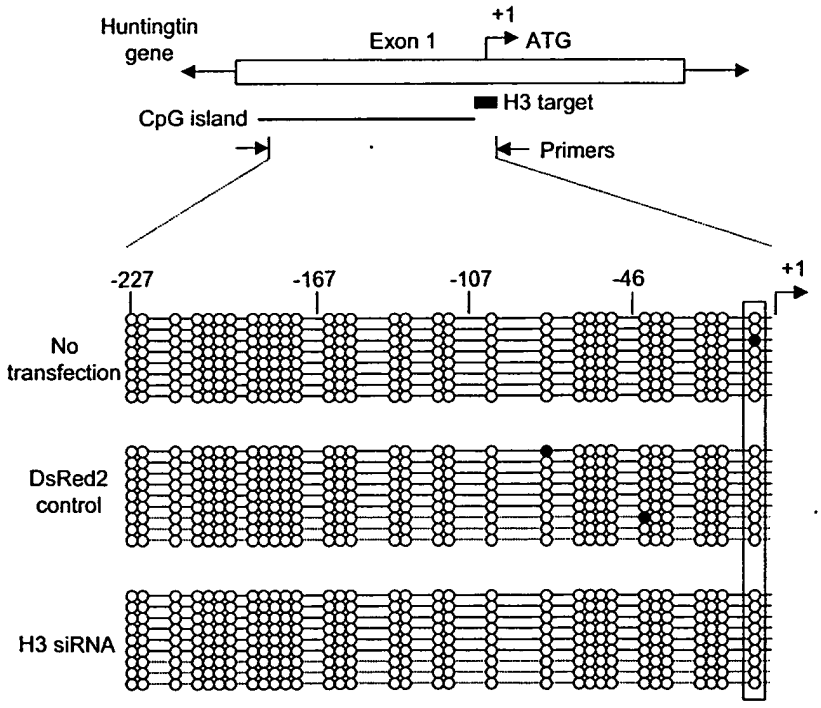

Fig. 2. CpG methylation at the target genomic site of H3 dsRNA. The schematic map of the local genomic region of the H3 dsRNA target site in the human huntingtin gene is shown at top. The H3 target site (black box beneath the map) in exon 1 (open rectangle) of the gene includes the initiation codon (ATG, bent arrow). The bisulfite PCR primers indicated by arrows below amplify a region including a CpG island (a thick line under the map) and the H3 target sequence (black box). Bisulfite PCR clones are represented by lines on which filled circles and empty circles indicate methylated and unmethylated CpGs, respectively. On top of the blocks of bisulfite clones, the distance relative to the initiation codon (+1, bent arrow) is indicated by numbers above the map. Dotted and solid lines represent the bisulfite clones derived from U-118 and U-87 cell lines, respectively. Each group of bisulfite clones is displayed as a block of lines with the construct used for cell transfection indicated on the left. The circles representing the CpG located within the H3 target sequence are enclosed by a long vertical box.

CpG methylation at the target genomic region of H5 dsRNA construct

We also analyzed the CpG methylation pattern of the H5 target genomic region. The 21 bp genomic sequence target of the H5 dsRNA is completely included within exon 4 of the human huntingtin gene. A single CpG is located in the H5 target genomic site, and a 104 bp CpG island is 305 bp 3′ of exon 4 (Fig. 3). Genomic DNA isolated from the U-87 human glioblastoma cell line transfected with H5 construct was subjected to bisulfite sequence analysis. DNA from untransfected U-87 cells or from U-87 cells transfected with the control DsRed2 plasmid vector was also analyzed. In contrast to the H3 target region, the genomic DNA within the human huntingtin H5 target region exhibited dense CpG methylation. The native DNA methylation pattern derived from the control, untransfected cells indicated that this region is heavily methylated in this human glioblastoma cell line. No significant difference in the CpG methylation pattern of this region was detected in the cells transfected with either the DsRed control or the H5 dsRNA-expressing construct pH5.

Thus, this study suggests that unlike plants, silencing dsRNAs might not involve RdDM at the target genomic region in cultured cells. However, it remains to be determined if this is a universal finding. Furthermore, a recent report demonstrated that DNA methylation is independent of RNA silencing in the fungi *Neurospora* [23], suggesting that although prevalent in plants, RdDM is not an absolute method of targeting de novo DNA methylation. In addition, dsRNA expression in mouse oocytes resulted in post-transcriptional silencing, but did not induce sequence-specific DNA methylation of the cognate DNA sequence [24]. Although no detectable change in the DNA methylation pattern in the human huntingtin gene locus was correlated with the expression of the dsRNAs, various chromosomal loci or tissue types could respond to siRNA in diverse ways for epigenetic modification. In fact, in fission yeast and *Drosophila*, components in the RNAi pathways are also involved in the formation of heterochromatin as well as localization of the key protein HP1 [25,26]. Given the enormous therapeutic potential of siRNA, it will be important to examine other possible epigenetic modifications associated with siRNAs in mammalian systems. Thus, the histone modifications that often accompany DNA methylation or heterochromatin formation should also be assessed in response to siRNAs or dsRNAs in mammals. However, the lack of RdDM by functional

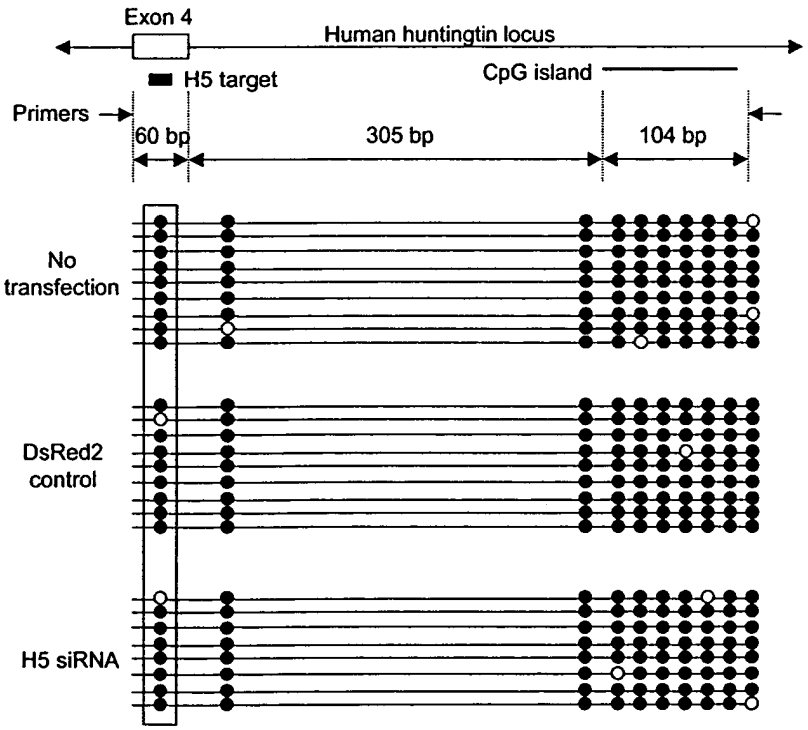

Fig. 3. CpG methylation at the target genomic site of H5 dsRNA. At top, the neighboring genomic region of the H5 dsRNA target site in the huntingtin gene is represented schematically. The target genomic site of the H5 dsRNA (black box) is entirely within exon 4 (exon 4, open rectangle) of the huntingtin gene. Exon 4 is ~60 bp in length, and a CpG island of ~100 bp (thick solid line below the map) is located 305 bp 3′ to exon 4. This CpG island and H5 target sequence (black box) were amplified using bisulfite PCR primers shown as arrows below. Bisulfite PCR clones are represented by lines on which filled circles and empty circles indicate methylated and unmethylated CpGs, respectively. All the bisulfite clones were derived from U-87 genomic DNA. Each group of bisulfite clones is displayed as a block of lines with the cell transfection status indicated on the left. CpG circles indicated by a long vertical box represent the CpG present in the H5 target genomic sequence.

siRNAs targeted to the huntingtin gene suggests that this mechanism of gene silencing may not invoke epigenetic modifications in human somatic cells. In addition, it underscores its potential use in the treatment of such a devastating dominant negative disorder as Huntington's disease.

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